THE SIGNIFICANCE OF MARINE BACTERIA IN THE FOULING OF SUBMERGED SURFACES

CLAUDE E. ZOBELL AND ESTHER C. ALLEN

Scripps Institution of Oceanography of the University of California, La Jolla, California

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The attachment and subsequent growth of the wildly promiscuous assemblage of visible plant and animal organisms on the hulls of ships and other submerged marine structures is known as fouling. Our present-day knowledge of the nature and extent of fouling is discussed comprehensively by Visscher (1928b). The vast economic loss resulting from fouling has instigated extensive investigations of its cause and more particularly of practical methods for its prevention. However, attention has been focused mainly upon the habits, life histories and tolerances to poisonous paints and metals of the macroscopic encroachers such as barnacles, mollusks, tunicates, hydroids, and bryozoans. Surprisingly, little, or no, attention has been devoted to the exact sequence of events, especially during the initial stages, to the relationship of one group of organisms to another, and to the associated microscopic life. It is the purpose of this paper to report the observations which have been made at the Scripps Institution of Oceanography at La Jolla during the last two years on the attachment of bacteria and kindred microörganisms upon submerged surfaces,[1] and to discuss their possible significance with reference to fouling.

The studies of Wilson (1925) at the Scripps Institution on marine algal successions indicated that colonial diatoms were the first

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sessile organisms to appear on submerged plates; but his methods of investigation made no provision for the observation of bacteria. Although he did not apply the procedure to the fouling problem Naumann (1925) reported that, due to their tenacious attachment, excellent preparations of iron bacteria can be prepared by the submergence of glass slides in iron-bearing waters. Both Hentschel (1925) and Thomasson (1925) submerged slides and later examined them microscopically to follow the distribution of diatoms and other minute sessile aquatic organisms, but neither the fouling problem nor bacteria were considered. Using a direct microscopic technique Henrici (1933) concluded from his studies on fresh-water bacteria that "it is quite evident that for the most part water bacteria are not free floating organisms, but grow attached upon submerged surfaces." He found that following the submergence of glass slides in lake water a deposit of bacteria soon becomes apparent and increases progressively until individual cells can be distinguished only with difficulty.

In preliminary observations on the nature and distribution of marine bacteria and their rôle in the fouling of submerged surfaces, ZoBell and Allen (1933), using a procedure somewhat similar to that employed by Henrici, indicated that numerous bacteria soon attach themselves to glass slides submerged in sea water and that bacteria, and to a lesser extent diatoms and actinomyces, usually precede the attachment of barnacles and other fouling organisms. This report is a continuation of those studies.

METHODS OF INVESTIGATION

Standard micro-slides were submerged off the end of the Institution's pier which extends one thousand feet seaward from shore. The slides were tied to a carrier (fig. 1) which consists of a piece of lead about 12 by 4 by 0.25 inches covered with wood, and the whole coated with paraffin. The heavy lead gives anchorage and stability, and the wood and paraffin keep the slides from direct contact with the metal. Grooved wood strips and cord string provide for fastening twelve to sixteen slides on the faces of the carrier. The device was suspended by means of a cotton rope in from six to twelve feet of water, depending upon

the tide. Within these limits the depth of submergence did not influence the results and neither did the tidal phase, the main effect of the latter being merely to change the depth of the water, because the pier virtually extends in the open ocean. Adherent

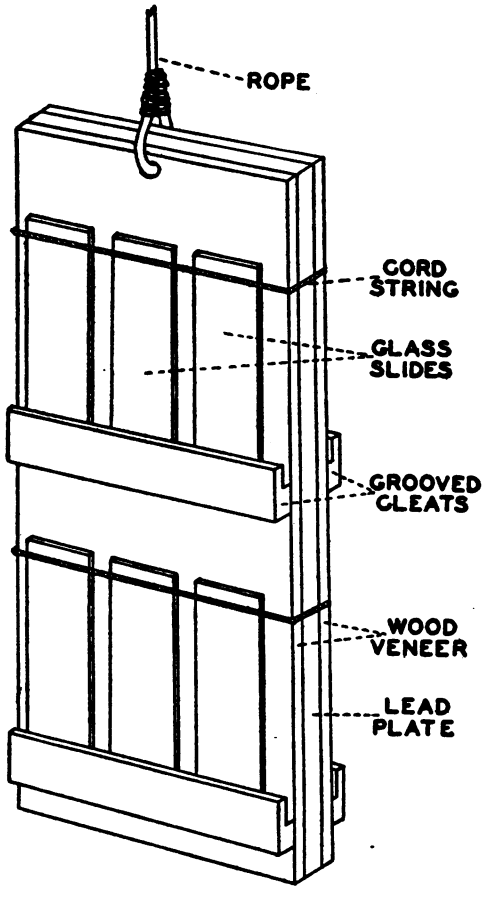

FIG. 1. WOOD COVERED LEAD CARRIER FOR HOLDING SUBMERGED GLASS SLIDES

growths were scraped from the carrier as often as required and the latter was sterilized by dipping in hypochlorite solution. Prior to submergence the slides were cleaned, wrapped in paper, and heat sterilized. Aseptic precautions were exercised in their

manipulation. They were exposed to the sea water for periods varying from a few hours to a few days. They were examined in the fresh condition and also after staining. Loeffler's methylene blue and Hucker's ammonium-oxalate crystal-violet are useful stains; but the most satisfactory results have been achieved with Conn's (1918) rose bengal consisting of 1.0 per cent of the dye and 0.02 per cent anhydrous calcium chloride in 5 per cent aqueous phenol solution.

In order to obtain cultures of bacteria that form films or those which attach to solid surfaces, some of the exposed slides were plated directly in sea-water agar after rinsing them in sterile water to dislodge all except the desired types. This procedure was practicable only in the case of those slides which had been submerged for a few hours, because later there were too many bacteria on the slides. In other experiments bacteria from the adherent films were brushed off with a cotton swab in sterile sea water and appropriate dilutions thereof were plated. The method had no quantitative significance but it furnished a means of obtaining pure cultures of the film-formers.

RESULTS

The majority of the organisms which are found attached to slides submerged in the sea for one to seven days are definitely microscopic in size and most of these are bacteria. Table 1 illustrates the kind of record which was kept and shows the number of bacteria, other microscopic forms, and larger organisms visible to the naked eye, found on the 2- by 1-inch exposed area of the slide after twenty-four hours' submergence. All of the macro-organisms were enumerated, whereas the number of bacteria and other microörganisms per slide were calculated by counting those which appeared in a representative number of fields (magnification 980× and 430×, respectively) and multiplying by a factor. Usually at least fifty fields were scrutinized.

Figure 2 shows graphically the number of bacteria by weekly averages which were found attached to slides which had been submerged for forty-eight hours during the first six months of 1933. There were millions of attached bacteria per 2 square-

inches of slide while concomitant plate counts revealed only hundreds of bacteria per cubic centimeter of water in which the slides were submerged. The lack of relationship between the number of marine bacteria found attached to slides and the number demonstrated by plating procedures is noteworthy.

Controlled laboratory experiments devised to simulate field conditions show that there are several factors besides the number of bacteria present in the sea water which influence the number found attached to glass slides submerged therein. In the first place, not all marine bacteria attach themselves to glass slides even under favorable conditions. This was revealed by testing

TABLE 1

Number of bacteria, other microscopic organisms and macroscopic organisms attached to 2 square inches of micro-slides submerged in the sea for 24 hours

DATE OF SUBMERGENCE	BACTERIA	OTHER MICROSCOPIC ORGANISMS	MACROSCOPIC ORGANISMS
February 7, 1933	2,820,000	3,500	0
February 13, 1933	1,860,000	500	0
February 14, 1933	1,260,000	8,900	0
February 20, 1933	600,000	500	0
February 27, 1933	9,060,000	1,000	0
March 6, 1933	7,800,000	10,300	1
March 9, 1933	6,720,000	12,000	0
March 15, 1933	3,240,000	400	1
March 20, 1933	1,920,000	300	0
March 29, 1933	4,260,000	600	0

73 pure cultures, differing physiologically or morphologically, which had been isolated at random by planting sea water (ZoBell and Feltham, 1934). These were cultivated in bottles of sea-water broth into which sterile glass slides were inserted vertically. After two days' incubation at 25°C. it was found by microscopic examination of the slides that only one-third, or 24 of the 73 cultures, were firmly fixed to the solid surfaces. However, three of the cultures were found attached only to solid surfaces, forming films of micro-colonies on the glass slides and the walls of the bottles, while the broth itself was not perceptibly turbid.

Micro-colonies seldom appear on slides exposed to sea water

for only a few hours; the cells for the most part occur singly or in pairs, or more rarely, in long chains. The paucity of microcolonies is attributed to the lack of available nutrients in sea water, because they appear abundantly on slides in sea water to which nutrients have been added. Under the latter conditions

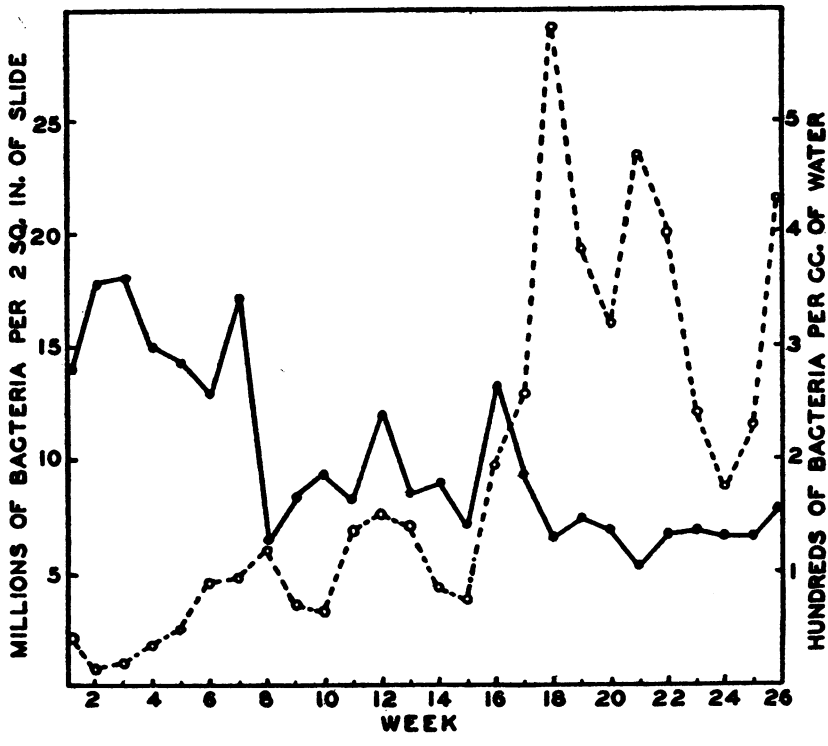

FIG. 2. Broken line represents millions of bacteria found attached to two square inches of submerged slide in forty-eight hours and solid line gives the average plate count per cubic centimeter of sea water for the first twenty-six weeks in 1933.

it is quite evident that the bacteria are multiplying on the surface of the slide and all stages of cell division can be observed. Ordinarily it requires two to four hours for appreciable numbers of bacteria to become attached solidly to glass slides. Firm attachment after coming into contact with the surface is not immediate,

as it seems to take several minutes for the bacteria to cement themselves to the glass. Thus, the submergence of a slide in sea water replete with bacteria, followed by its immediate removal, does not result in the attachment of bacteria, although a few may adhere temporarily. But let the slide remain submerged for an hour or two, and if the bacteria are of the attachment type and are in the logarithmic phase of growth, they will be found profusely, so firmly glued to the slide that running water will not detach them.

Various sizes and shapes of bacteria appear. Ovoid organisms with a diameter of less than 1 micron are most numerous. Such coccobacilli comprise at least 60 per cent of the total number of bacteria. Slender bacilli, 1μ to 2μ in length, are common. Larger rods occur less frequently. True cocci and spirilla are seldom seen. The majority of these organisms possess well-defined capsules which in some cases are twice or three times the size of the enclosed bacterium. Nearly all of the bacteria are Gram-negative.

Assorted filamentous forms occasionally appear. These are of two principal types: (*a*) actinomyces, consisting of small patches of slender mycelial threads which appear both continuous and fragmented, and (*b*) larger, straighter, branched filaments which are probably Chlamydobacter. Leptothrix has been observed.

While it is impossible to identify species of bacteria from a consideration of morphological features only, it is estimated from the diversity of form, size and structure, that no less than forty or fifty species of bacteria are represented regularly, and probably more. Twenty-eight pure cultures have been isolated from twenty-four-hour films for further study. Immediately following isolation the majority of these grow readily in sea-water media, but not in a corresponding nutrient solution prepared with fresh water, thereby indicating their halophilic specificity. Several of these have been completely characterized according to the methods of the Committee (1930) on the Pure Culture Study of Bacteria, and they are found to be new species.

Three representative species which have been encountered frequently attached to submerged surfaces are herewith described.

No. 577. *Achromobacter marinoglutinosus*, n. sp. Short Gram-negative rods 0.7 to 1.0 by 1.8 to 2.4 microns with rounded ends. Methylene blue shows granular structure. Occur singly, in pairs, and in clumps. Encapsulated. Motile by means of polar flagella.

Gelatin stab: Moderate filiform growth with slight napiform liquefaction. No pigment.

Agar slant: Moderate, filiform, flat growth. Butyrous consistency.

Agar colonies: Round with concentric circles and crinkled radial line, 1.5 to 5.0 mm. in diameter. No pigment.

Broth: Moderate clouding, marked ring, adherent film of growth on test-tube wall, and flaky sediment. No growth in milk or on potato.

No indol.

Produces hydrogen sulphide and ammonia from Bacto-tryptone.

Reduces neither nitrate nor nitrite.

Does not ferment glucose, lactose, sucrose or mannitol.

Produces acid but no gas from xylose and dextrin.

Starch hydrolyzed.

Facultative aerobe.

Optimum temperature, 20° to 25°C.

No. 580. *Achromobacter membranoformis*, n. sp. Rods 0.9 to 1.2 by 3.5 to 4.8 microns, occurring singly and in pairs. Encapsulated. Motile by means of lophotrichous flagella.

Gelatin stab: Filiform growth, best at top with slow crateriform liquefaction.

Agar slant: Moderate, beaded, raised growth. Membranous consistency. Becomes browned with age.

Agar colonies: Circular 1.0 to 2.5 mm. with crinkled surface.

Broth: Slight clouding, flocculent sediment, film of growth on walls of test tube.

No growth in milk or on potato.

No indol or hydrogen sulphide.

Reduces neither nitrate nor nitrite.

Produces acid without gas from glucose, sucrose, dextrin, and mannitol. Lactose and xylose not fermented.

No diastatic action.

Aerobic.

Optimum temperature, 20° to 25°C.

No. 588. *Flavobacterium amocontactus*, n. sp. Slender Gram-negative rods, 0.4 to 0.7 by 1.6 to 2.3 microns, with rounded ends. Stain

very lightly. Occur singly or in irregular clumps. Possess well-defined capsules. Actively motile by means of peritrichous flagella.

Gelatin stab: Good filiform growth with rapid saccate liquefaction.

Agar slant: Abundant, filiform, smooth, glistening bright yellow growth having a butyrous consistency. Originally liquefied agar but this property was lost following artificial cultivation.

Agar colonies: Circular 2.0 to 4.0 mm. in diameter, yellow.

Broth: Good growth in sea-water broth with ring at surface, strong clouding and abundant viscid sediment. No odor.

No growth in milk.

No growth on ordinary potato but slight yellow growth on potato dialyzed in sea water.

No indol.

Produces hydrogen sulphide.

Ammonia liberated from peptone.

Reduces both nitrate and nitrite.

Does not ferment glucose, lactose, sucrose, xylose or mannitol.

Starch not attacked.

Facultative aerobe.

Optimum temperature, 18° to 21°C. Optimum reaction, pH. 8.0.

The microscopic organisms other than bacteria which become attached to the submerged slides consist chiefly of diatoms, the common genera being Grammatophora, Navicula, Licmophora, Fragilaria, Striatella, and Nitzschia. Although the diatoms are much less numerous than bacteria, they are always more abundant than the macroscopic organisms. The data summarized in table 2 show that approximately a hundred times as many bacteria as of all other classes of organisms combined were attached to the submerged slide during the primary stages. Furthermore, it is of interest to note that the accumulation of bacteria and, more particularly, of diatoms does not proceed in arithmetical progression with twenty-four-hour intervals; or, in other words, when the number of attached organisms is plotted against time, a parabolic curve rather than a straight line results. This indicates either a multiplication of the attached organisms or a favoring influence of the film-formers upon subsequent attachment. Both factors are probably operative.

As indicated by table 2, very few macroscopic organisms, or

those which could be located without the aid of a lens, were attached to slides which had been submerged for only three days. Their number continued to increase slowly but progressively from the fourth to the seventh day, the longest submergence period considered in these studies. Suctoria and hydroids were most abundant, both appearing with regularity throughout the year but in appreciable numbers only after five days. Cyprid larvae of barnacles were found occasionally from March to August, being common only in the last four months of that period. Most of the larvae were not firmly attached to the slides in seven days and were readily washed off. Bryozoa appeared very rarely. For more information concerning these macroscopic sedentary marine organisms in this vicinity the reader is referred to Coe (1932).

TABLE 2

Average number of different classes of organisms, which were attached to 2 square inches of slide after 24, 48, and 72 hours' submergence during the first six months of 1933

PERIOD OF SUBMERGENCE	BACTERIA	OTHER MICROÖRGANISMS INCLUDING DIATOMS	MACROSCOPIC ORGANISMS
hours			
24	2,023,800	2,560	0.3
48	9,268,200	10,840	1.2
72	24,115,400	28,310	1.9

What is the relation of the primary bacterial film to the attachment of other forms? To elucidate this point further, film-coated glass slides were submerged concurrently with sterile slides as controls. The films were prepared in the laboratory by placing slides in bottles of nutrient broth inoculated with cultures of attachment bacteria until a good film had formed. Following one to five day's submergence in the sea the film-coated and the originally sterile slides were examined for attached organisms. The film-covered slides had a noticeably greater number of attached organisms than did the slides which were originally sterile. Table 3 presents the average results.

Not only do bacteria play an important rôle as primary film-formers, but they are also found in abundance associated with the

growths on fouled surfaces during the later stages of fouling. Several specimens of slimy film were scraped from the hull of the U. S. N. Destroyer LAUB as it was being dry-docked. The vessel had been in the water for thirty-eight months. Direct microscopic analyses by a modified Breed and Brew (1916) method of this material properly diluted revealed that it contained from a few million to several billion bacteria per gram of moist scrapings. From preliminary results which will be reported in greater detail elsewhere it is estimated that, on an average, 8 to 9 per cent by volume of the fouling cumulation on this particular vessel consisted of bacteria. This plethora of bacteria is attributed to the abundance of organic matter on which they feed and to their sedentary properties.

TABLE 3

Average number of microörganisms (excluding bacteria) and macroscopic organisms attached to 2 square inches of sterile micro-slides and to slides coated with a bacterial film under comparable conditions

PERIOD OF SUBMERGENCE	MICROÖRGANISMS		MACROÖRGANISMS	
	Sterile slides	Film-coated slides	Sterile slides	Film-coated slides
hours				
24	15	42	0.3	5.4
48	23	89	1.1	7.2
72	98	276		
120	852	1,257	18.6	43.8

Hilen (1923) reported that the slime which forms on surfaces in ocean water is "composed of a variety of bacteria as well as yeasts and molds." Corroborating these studies, Angst (1923) found that the slime on ships' bottoms is caused to a large extent by bacteria, and, furthermore, he concluded that "the slime bears a direct relation to the appearance of the barnacles."

CONCLUSIONS

Our observations show quite conclusively that bacteria and, to a lesser extent, other microörganisms are the primary film-formers on submerged glass slides, and that such films favor the subsequent attachment of the larger and more inimical fouling

organisms. The film of bacteria may promote the attachment of macroscopic organisms in different ways. They may form a mucilaginous surface to which the fouling organisms in the planktonic or free-swimming stage readily adhere until they can prepare their own holdfast. Again, the bacterial film together with the particulate organic detritus which sticks to it, may furnish the fouling organisms with a suitable source of food during their infancy. Speaking of barnacle larvae in captivity, Visscher (1928a) says, "These organisms have been observed to 'walk' for considerable distances, and have been seen to 'test' various areas for a period of more than an hour before finally attaching." It is possible that at least a part of this delay in attaching may be due to the influence of microörganic films. Furthermore, while it is still a matter of conjecture, it is entirely possible that in the case of submerged surfaces which contain substances poisonous to the fouling organisms, the bacterial film forms a protective coating.

While it remains to be proved that barnacles, bryozoa, hydroids and other macroscopic fouling organisms will not attach without the aid of a primary bacterial or other microörganic film, the foregoing studies suggest that microörganisms merit considerable attention in investigating the exact cause and ultimate prevention of fouling.

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